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Cyclotides Discovery, Distribution, Structure and Biological Activities

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Abstract

Many plants of the Violaceae plant family have been used in traditional remedies, and these plants often contain cyclotides, a particular type of plant cyclopeptide that is distinguished by a cyclic cystine knotmotif. Cyclotides are small disulfide-rich peptides that are characterized by a head-to-tail cyclized peptide backbone and a knotted arrangement of three conserved disulfide bonds. They are present in many plants from the Violaceae, Rubiaceae and Cucurbitaceae families, with individual plants expressing a suite of dozens of cyclotides. They are divided into 3 sub-categories recognized by structural feature and sequence of amino acids. The two fundamental subfamilies named bracelet and Mobius cyclotides, include sequences which are moderately preserved inside, yet not among the subfamilies. Their primary function in plants is thought to be as defense agents, based on their potent insecticidal activity, but they also have a range of other biological activities, including anti-HIV, antimicrobial and cytotoxic activities. The sequence of the gene will give us more information regarding its structure and other physicochemical properties, as the protein function is determined by its structure so to put some light on its function. The discovery of cyclotides and their gene sequence describe their unique structural features and a range of bioactivities that would be useful for pharmacological applications.

Keywords : Cyclotides, Disulfide-rich peptides, Bracelet, Mobius Cyclotides, Traditional Remedies

Introduction

Cyclotides are naturally occurring plant proteins containing about 30 amino acids with a unique feature of head to tail cyclized backbone. Three conserved disulfide bonds are also present in a knotted arrangement [30]. Two of the disulfide bonds (CysI–CysIV and CysII-CysV) and their connecting backbone segments form a small embedded ring that is penetrated by the third disulfide bond (CysIII–CysVI). This unique structure is known as a cyclic cystine knot (CCK) motif [4]. Their three-dimensional structure of prototypic cyclotides, kalata B1 is represented in Figure 1 [62]. In mid-1990s the presence of cyclic proteins of this size was hindered but now they are known to man for nearly two decades [11]. They do not have N- or C-terminus as all amino acids of the protein are linked in peptide bonds' continuous cycle [60].

The prototypical cyclotides, kalata B1, was discovered in a traditional Congolese medicine "kalat-kalata" by Lorents Gran, a Norwegian doctor, in the 1970s. Derived from a boiled leaf extract of the herb *Oldenlandia affinis* DC. (Rubiaceae), this preparation was used to accelerate childbirth [22]. One of the active uterotonic agents, kalata B1, was later characterized as a 29 amino acid cyclic peptide and its three-dimensional structure elucidated using NMR spectroscopy [62]. This discovery led to efforts to find similar examples of cyclic peptides in other plants, and the term "cyclotides" was defined in 1999, when it became apparent that related macrocyclic peptides were present in a large number of plants [11]. A further 20 cyclotides have

since been reported in O. affinis, [13] and ~400 cyclotides have been sequenced from a variety of other plants (www.cybase.org.au) [40]. More recent pharmacology analysis has confirmed the uterotonic activity of *O. affinis* extract, including kalata B7, for inducing strong contractility on human uterine smooth muscle cells [34]. There were no reports published on kalata B1 for another 20 years, until we described the sequence, circular backbone and three dimensional structure, including the cystine knot topology [62].

Viola tricolor L., a typical green plant, belongs to the Violaceae family, has been utilized in drug for relieving coughs, heat clearing and detoxification [57]. Arrangement and utilization of the drug were encompassed by a level of mystery and well along reports (Gran, individual correspondence) demonstrated that essence may be connected straightforwardly to birth channel as options in contrast to being gulped. Regardless of its method of use, decoction significantly affected uterine compressions and prompted speedier delivery. Hearsays of its orally active in ethno-medicinal situations proposed that heat and oral stability of active constituents. Kalata B1 was decontaminated from concentrates of plants and recognized as a functioning uteronic mediator [62]. but, after 1995, primary structure of peptides was affirmed.

Before the discovery of these cyclotides, the cyclic proteins were realized as big as 15 amino acids [11]. Albeit linear types of cyclotides do present in plants, they are not that common [41]. Although they exhibit small size but comprise of features of larger proteins like defined secondary structure and a compact three dimensional fold [60].

Cyclotides are cysteine-rich plant peptides encoded by genes and are synthesized by ribosomes. The peptide contains 28-37 amino acids, the unusual characteristic for single peptide bond and three disulfide bonds having cyclized backbone [15].This structural motif has already been found in different species like plants, fungi, marine molluscs, spiders and insects [12]. This article provides a brief overview of their properties and their use as molecular frameworksfor the design of peptide-based diagnostic and therapeutic tools.

Diversity and Distribution of Cyclotides

They are divided into 2 subcategories recognized by based on the presence or absence, respectively, of a conserved Cis-Pro residue in loop 5 [12]. These two fundamental subfamilies, include sequences which are moderately preserved inside, yet not amongst the subfamilies [61]. The subfamilies are further differentiated by fluctuations in loop sequence and size, in addition to a net charge. Members of Bracelet subfamily generally have a higher net positive charge due to the presence of residues like Lys and/or Arg in loops 5 and 6 while other Mobius cyclotides have no charge or have few charged residues [7].

In contrast to these two subfamilies, a six-residue loop 1 is present in third subcategory, i.e. trypsin inhibitor subfamily from squash family. Two macro-cyclized peptides M. C. trypsin inhibitor II (MCoTI-II) and M. cochinchinensis trypsin inhibitor I (MCoTI-I) are examples of cyclic trypsin inhibitors isolated from the seeds of Momordica cochinchinensis, a tropical vine belonging to the Cucurbitaceae family [29]. Specifically, MCoTI-I and MCoTI-II are 34 amino acid trypsin inhibitors that contain six Cys residues arranged in three disulfide bonds. These were different from the earlier cystine knot macrocyclic peptides in that they had a different spacing of the Cys residues [41]. These are alluded to cyclotides as structurally complex small proteins because of the 3D topology and the way they are gene encoded [31].

Plant producing cyclotides usually represent a few, to more than a few hundred, diverse cyclotides [49]. They likewise inclined to be thermally steady and naturally have well defined topology and in this manner pay a lesser entropic price for sticking to a receptor [14]. Viola is dispersed in both the southern and northern temperate areas along with tropics and owns high variety with 585–625 species [62]. Most of cyclotides as of late found are in family of Violaceae, comprises of 30 thousand distinctive cyclotides [65]. The Violaceae family comprises 25 genera ([13], however, only 6 species (Melicytus species, Viola species, Gloeospermum species, Hybanso species, Leonia species and Rinorea species) were found to encompass

cyclotides [4]. Rinorea comprises of 225-275 species and 2nd largest genus of Violaceae family [62]. Fabaceae is the 3rd largest family around worldwide, that's its innovation symbolized a significant novel growth and this family consists of 18000 plant species from which many species are involved in human nutrition, like peas and beans. Only ten years ago, Cyclotides plants were officially documented as a family [11].

Small peptides obtained from microorganisms that have been known very well for a large time are not equivalent to these plant peptides known as Cyclotides. These are just 5–10 amino acids long, absent disulfide bond, and do not synthesized by ribosomes. There just are limited instances of bigger cyclized proteins inferred from microbes including a 21 amino acids peptide from Escherichia coli [3] and a 70 amino acids linear protein from *Enterococcus faecalis* [38]. Both deficient in disulfide bonds yet give off an impression of being gene encoded products.

Non-ribosomally synthesized cyclized peptides for example cyclosporin and ribosomally synthesized cyclized proteins for example, gassericin A [32], circularin A, closticin [33], bacteriocin AS-48 [21] and pilin proteins from microorganisms have been known for some time. Current discoveries of circular proteins from higher organisms, including the cyclotides [11], sunflowers trypsin inhibitor -1 [37], rhesus theta defensins -1 from primate leukocyte [57] display that higher organisms also comprise machinery required for regeneration of cyclized proteins.

Initial discoveries of cyclotides were made from Violaceae and Rubiaceae families, but currently two macro-cyclized peptides, M. C. trypsin inhibitor II (MCoTI-II) and M. cochinchinensis trypsin inhibitor I (MCoTI-I) were exposed from the Cucurbitaceae family. Two MCoTI peptides make another subfamily mentioned to as trypsin-inhibitor cyclotides [9].

There is ongoing interest in both discovering new cyclotides sequences as well as understanding the distribution of known cyclotides sequences to better understand their biosynthesis, evolution and range among plant species, and potential for agronomic and therapeutic applications [34]. Recently, combined proteomic and transcriptomic analytical approaches have been applied in large scale chemical screens for cyclotides to expand knowledge of their occurrence, sequence diversity, and phylogenetics [1].

A recent investigation of over 140 species selected from the majority of Violaceous genera highlighted the ubiquity of cyclotides therein and also the widespread representation of selected cyclotides among them, kalata S (Varv A) being present in 70% of the analyzed species [4]. Analyses of Viola species collected from regions in all continents sampled including Asia, South-East Asia, Europe, South America, Africa, and Australia have also supported the ubiquity of cyclotides among Violaceae [42]. Thus, to accelerate discoveries on the origins and evolution of cyclotides, it is of particular interest to focus on the distribution of cyclotides from the "sporadic" cyclotides-containing families, while continuing to capture data on Violaceous species endemic to isolated geographies [1].

Structure

Until recently, few naturally occurring acyclic derivatives of cyclotides had been found in plants. Acyclic permutants of, [18] cyclotides have been chemically synthesized and it is generally observed that linearization of the peptide backbone reduces or eliminates the biological activity of the native [2] cyclotides. However, recently a number of naturally [48] occurring "a cyclotides" have been reported in plants.

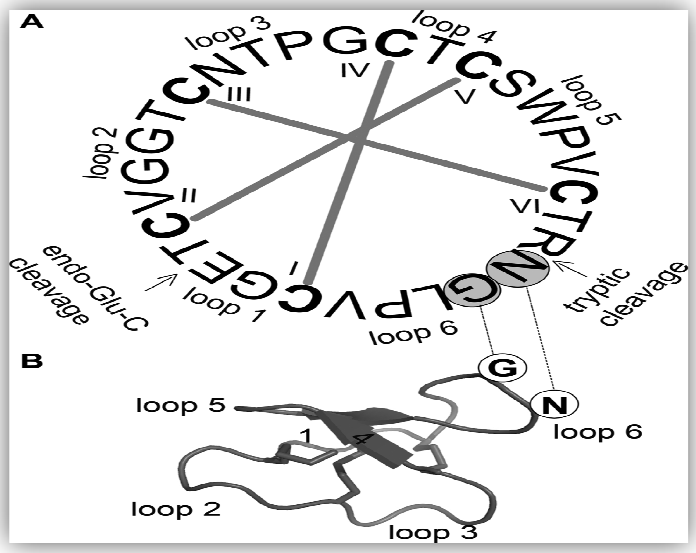

Figure 1. Sequence and three-dimensional structure of the prototypic cyclotides kalata B1. (A) The amino acid sequence of kalata B1 showing the six Cys residues (labeled I–VI), the disulfide connectivity, and the cyclic backbone. (B) Three-dimensional structure of kalata B1 (Protein Data Bank ID code: 1NB1).6 The segments between two adjacent Cys residues are termed loops and are numbered 1–6. The circular backbone is formed by a peptide bond in loop 6 between Gly-1 and Asn-29. Potential protease cleavage sites of reduced cyclotides, which are used in sequencing studies, are indicated by arrows.

The three-dimensional structures of five native cyclotides (kalata B1, circulin A, cycloviolacin O1, palicourein and MCoTI-II) have been published [2]. A highly conserved framework is present in the known structures, as shown with three representative structures in (Fig. 3).

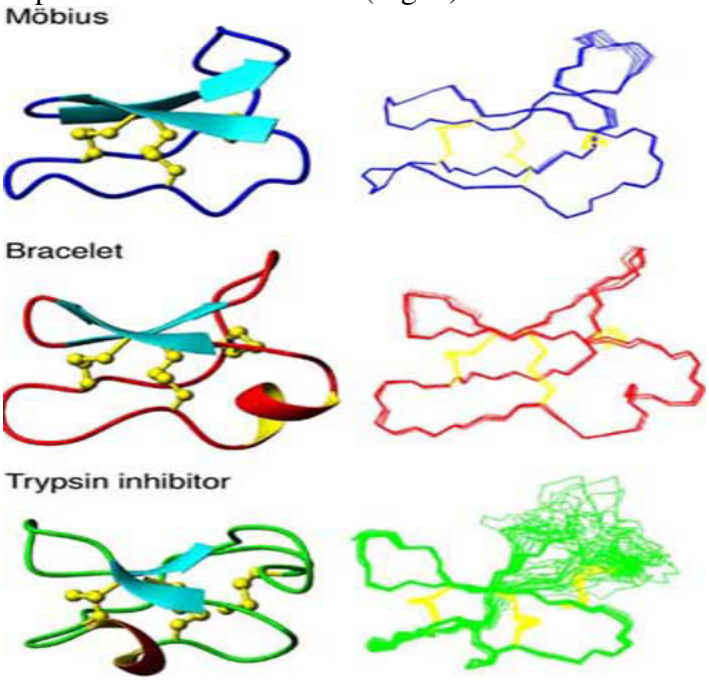

Fig. (3). Solution structures of kalata B1, cycloviolacin O1 and MCoTI-II. These peptides represent the Möbius, bracelet and trypsin inhibitor cyclotides, respectively. Ribbon diagrams are shown on the left and the disulfide bonds are shown in ball and stick format. Overlays of the 20 lowest energy structures are shown on the right of the diagram, with the disulfide bonds shown in yellow. Both kalata B1 and cycloviolacin O1 have well defined structures over the whole molecule, whereas MCoTI-II has a disordered loop 6.

The relatively small size of the cyclotides together with their circular backbone and three disulfide bonds suggests that this overall fold will be present throughout the family. On this basis, the structures of several other cyclotides, including cyclopsychotride A [55], vodo N and vodo M [54] have been modeled.

The cyclotides structures are characterized by a cystine knot arrangement of the disulfide bonds and a b-sheet secondary structure. The cystine knot involves the bonds between Cys^{I}-Cys^{IV} and Cys^{II}-Cys^{V} and their intervening backbone sequences forming a ring through which the third disulfide bond (Cys^{III}-Cys^{VI}) threads. It is this cystine knot arrangement coupled with the circular backbone that defines the cyclic cystine knot motif [11]. The secondary structure b conserved throughout the cyclotides involves a–hairpin centered in loop 5. This hairpin is associated with a third b–strand, but this strand is usually distorted from ideal geometry to the extent that it is generally not formally recognized as part of a triple stranded b–sheet in secondary structure analysis programs. In the bracelet cyclotides a small region of helix is present in loop 3, in contrast to the rather extended structure of this loop found in the Möbius and trypsin inhibitor cyclotides. MCoTI-II contains a g-turn in loop 2 and the overall fold is highly homologous to that of acyclic forms of the squash trypsin inhibitors [20]. Interestingly, loop 6 of MCoTI-II appears to be very flexible, suggesting that in this case cyclisation is not present to stabilize the fold but perhaps may be present to confer resistance to exo-protease attack by removing the N- and C termini.

Regardless of these different activities, the regular job of cyclotides is defensive, hypothesis supported through perception; kalata B1 restrains development as well as growth of *Helicoverpa punctigera* larvae in bolstering trials [31].

Potential of Cyclotides in Pharmacology and Drug Development

Recent cyclotides research has focused on the use of these peptides in drug discovery applications. Their potential for such applications stems from their extraordinary stability against chemical denaturants, high temperatures, or proteases, as well as their natural sequence diversity, biological activities, and amenability to peptide engineering via sequence substitutions.

In old times, investigator observed the properties of plant *Viola tricolor* for their non-peptidic parts, like flavonoids portions were observed for their anti-bacterial, anti-inflammatory and antioxidant actions extracts [64].

Flavonoid compounds are used as antioxidant actions for biological actions like heartsease's parts. These compounds are more significant due to its metabolites activities and helpful in the treatment of cardiovascular diseases, liver diseases, prophylaxis, inflammations, immune disorders and many other problems [27].

For the treatment of many skin diseases like pain, inflammation and burn injuries are cured by the plant "Heartsease (*Viola tricolor* L.)" [8]. In older times in the country of Europe, it was proved as effective remedy for the treatment of inflammation in lung infections, inflammatory skin problems and atopic dermatitis [25]. The herbal extract of *Viola tricolor* is used to treat the inflammatory disease (psoriasis) [51].

Many studies revealed the importance of phytotherapy as herbal medicine which is declared in many books [16] and these species consists of many compounds like ascorbic acid, tocopherol and saponins. Heartsease plant is recognized as a plant containing flavonoids, catechins, salicylic acid, polysaccharides,

cumarins and phenyl carbonic acids [16]. An anti-oxidant flavonoid compound present in *Viola tricolor* and act as biological activities [47].

Three cyclotides have been recommended and established as potent cytotoxic action. Cyclotides have been used as immune suppressive peptides which ceased the amplification of T-lymphocytes [23].

This plant extract suppresses the propagation of activated blood mono-nuclear cells [28]. Previously, cyclotides are considered as accountable to suppress the cancerous cell propagation [54]. Recent studies reported the findings of vigno 1–10 from *Viola ignobilis* [26], vigno 5 biological activity was examined to context on cervical cancer in human body. The mission to expose new therapeutic compounds for cancer treatment and administration is a never-ending project. In present age, the anti-cancerous action of cyclized peptides has involved a lot of concentration [4].

Earlier studies target to explore value of Cyclized Cysteine Knot scaffold in the field of drug design tangled in inspecting the capability of scaffold to give place to foreign sequence replacements. These two referends of kalata B1, containing polar residues were replaced for residue that make portion of hydrophobic covering in loop five were manufactured [15].

The hydrophobic covering is supposed to have an important effect on foldaway but these two altered cyclotides referends reserved natural fold. Besides, changed peptides required unwanted hemolytic action of this kalata B1. Discovery established lenience of cyclotides outline to residue replacements and significantly specified that comparatively negligible sequence dissimilarities can remove undesirable toxic actions including hemolytic action [15].

Cyclotides as Immunomodulators

One newly recorded biological activity of cyclotides is immunomodulation. To position this type of work in framework we noticed that a depsipeptide cyclosporin A, is a medically utilized immunosuppressive medication [39]. Cyclized peptides obtained from vegetations have as well engrossed consideration with respect to their possible usage as immunosuppressants. From *Linum usitatissimum* Cyclolinopeptide A was initial orbitide assumed to have activity of an immunosuppressant [62] Cyclotides separated from *O. affinis* and *V. tricolor* [23] also have reported immunosuppressant properties [59].

A cyclotides namely Kalata B1 obtained from *O. affinis*, is well characterized immunosuppressant cyclotides up to now. In vitro natural Kalata B1 prevents the growth of human lymphocyte, and structure-activity relationship investigation displayed that certain amino acid substitution on its biologically active exterior, i.e., V10 K and T20 K, decreased and enhanced this activity, correspondingly [23].

Activities of Immunomodulation were detected from cyclotides extract of *Viola tricolor* when occurrence of Viola cyclotides efficiently inhibited human lymphocyte's growth and prevented stimulation of pro-inflammatory cytokine (interleukin 2) [59].

Original mechanism of cyclotides interrelation with molecular marks stay indefinable up to now, though, a current SAR examine of kalata β7 recommended that it's biologically activity in endorsing uterotonic shrinkage includes communication with vasopressin and oxytocin V1 G proteins-coupled receptor (GPCRs) [34]. Sequence resemblance among loop 3 in kB7 and oxytocin maybe elaborate why kB7 can play a role as a GPCR agonist in an operational receptor stimulation essay.

Generally, these new discoveries of cyclotides as an immunomodulant and with capacity to interrelate with GPCRs inspire more examination of their varied biological activities and latent as GPCR ligand in drugs design [14].

Joining investigations on linked straight cystine-knot proteins have emphasized latent of these small-proteins as layouts in drug design. Though they do not have fairly steadiness of CCK outline, they make valued outlines for emerging proof-of-concept examines and might be beneficial as remedial treatment of

functions where stability necessities are not too much strict. One example includes joining platelet accumulation inhibitory action onto EETI-II [50], which is a direct homologue of CCK molecules MCoTIII.

Alignment of Cyclotides in Membranes

A typical feature of cyclotides is a superficial patch of hydrophobic residue and this element, together with broad range of actions detected for different cyclotides, has prompted the proposal of a typical mechanism of activity including targeting of natural membranes [10]. Given this recommendation, current structural investigations have portrayed connections among cyclotides and membrane imitating DPC micelles [53].

Both uteronic peptides kalata B7 and kalata B1 have been appeared to communicate with DPC micelles and in spite of the fact that they stay on micelle exterior, they embed their hydrophobic covering inside micelle location, as displayed by explicit broadening of certain residues upon adding to paramagnetic probes 5 and 16-doxylstearate to this [17]. Once fused into micelles these probes are specially found near surface and core correspondingly, and by relating the reducing signals on micelle-linked peptides by every one of them, a clear image of position of peptides in micelle can be derived. **Figure 6** demonstrates the structures of kalata B7 and kalata B1 that are bound to micelle, and remarkably, demonstrates that they tie in different directions [53].

Difference in the manner they enclosed inside micelle is no doubt because of the extra positive charge in loop six of kalata B7 influencing local relations with micelle main groups, and varying surface uncovered hydrophobic patches that are essential membrane restricting sites on cyclotides. In spite of the fact that it is hard to sum up other cyclotides, at this phase it appears to be likely that cysteine knots of cyclotides do not control membrane connections but instead gives a central framework onto which placing of surface displaying hydrophobic covering directs binding collaborations.

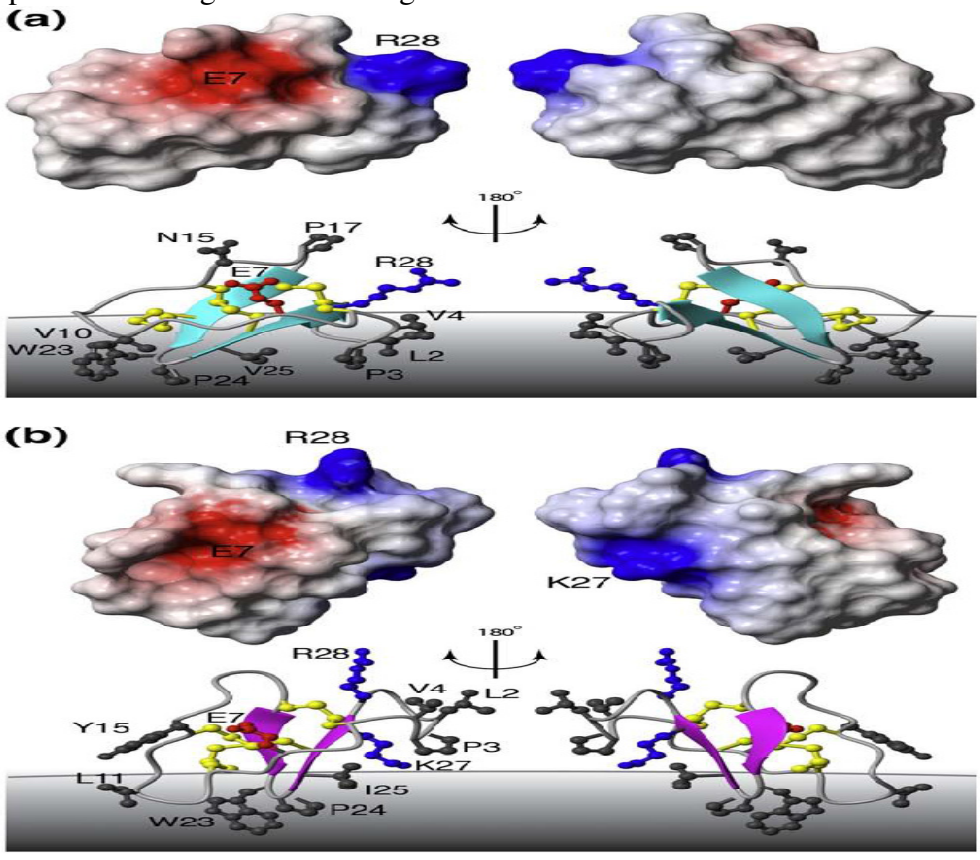

Fig. 6. Membrane tied to cyclotides. Nuclear Magnetic Resonance assemblies of (a) kalata B1 and (b) kalata B7 resolute in the occurrence of DPC micelles. External charges are revealed in red and blue for negative and positive charges. Main amino acids' side chain is displayed in ball and stick model and categorized with residues number and solo letters amino acid codes. Lower sheets demonstrate the positioning in association to micelle, which is revealed in grey, on foundation of reduction of signs after adding of paramagnetic probe. Views to right are interchanged 180° in association to view of the left. Remember that diverse cyclotides position varying in membranes, in spite of having a preserved cystine-knot center.

Though not any bracelet cyclotides has been considered in a membrane setting to date, prominent difference in their superficial characteristics from Möbius family, with loop five being exceedingly polar and loop three hydrophobic, recommends that they probably are to embed into membranes in an altogether different direction from Möbius cyclotides, for example, kalata B1 and B7. Ongoing investigations have discovered that cyclotides tie Mn^{2+} ions (and it was proposed that this could be vital for giving a positively charged exterior, progressively reasonable for associations with oppositely charged membranes [53]. Efficient significance of metal binding, assuming any, isn't yet known.

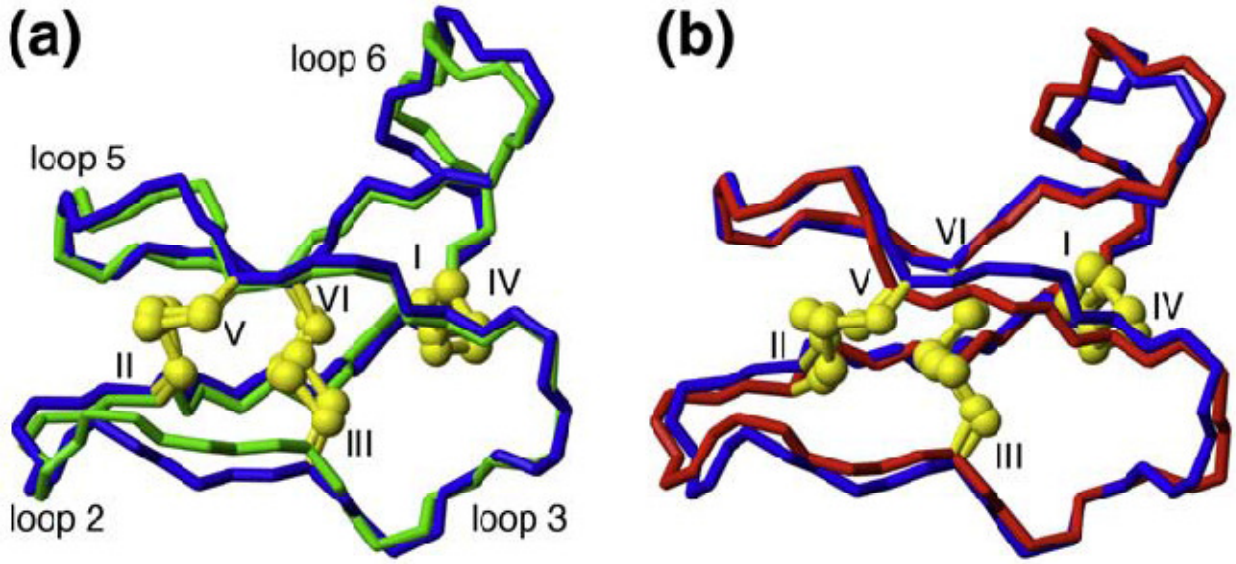

Figure 7:Comparison of structures in different environments.

(a) Framework of varv F Solution Nuclear Magnetic Resonance arrangements and X-ray topology of varv F [63].

(b) Framework of agent kalata B1 liquid Nuclear Magnetic Resonance arrangements and agent kalata B1 Nuclear Magnetic Resonance structure tied to DPC micelles.

Liquid structures are displayed in blue color while X-ray topology and micelle-linked structure are displayed in red and green.

Clear structural information on cyclotides was resulting from NMR solution of separated cyclotides after investigations. An underlying concern when investigations separated proteins in foreign settings is whether structural information signify the true functional form or structural alterations are instigated on membrane insertion or receptor binding. However, with inimitable cross braced structure of cyclotides, negligible fluctuations are predictable throughout these binding connections, and current information specify that this is situation. Figure 7 displays an evaluation of solution and clear assemblies of varv F and a contrast of the solution and micelle certain assemblies of kalata B1.

Conclusion

Currently, >700 cyclotides are deposited in CyBase. This family of cyclic plant peptides has been widely investigated as drug candidates and/or drug scaffolds, mainly because of their remarkable stability. Indeed, cyclotides have been characterized as self-defense molecules in plants, leading to their application as pesticides and antimicrobial agents. In addition, the study of cyclotides as scaffolds for the generation of improved variants (grafted cyclotides) with specific activities has attracted attention in alternative areas, including cancer, angio-genesis, inflammatory pain treatment, cardiovascular diseases, and cellular targeting.The prospect of developing a cyclotide-based drug or a cyclotide based insecticide in the near future is high. We can expect that cyclotides will shortly become active ingredients for many pharmaceutical products. Indeed, some questions regarding the role of cyclotides in plants remain, as well as cyclotide engineering for novel biological activities on humans. However, over the past decade, research on cyclotides has emphasized these molecules as orally effective, and with the potential to be produced by advanced technologies aiming at reduced costs. Moreover, cyclotides can also be expressed in plants. Thus, considering all the advantages and disadvantages of cyclotides, cyclotides represent fascinating molecules that offer a huge potential in many fields, including biomedicine, agriculture, and biotechnology.

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